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Phenytoin potentiates interleukin-1-induced prostaglandin biosynthesis in human gingival fibroblasts

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1 The effect of phenytoin (PHT) on prostaglandin E_2 (PGE_2) biosynthesis in human gingival fibroblasts stimulated by interleukin-1 (IL-1α, IL-1β) or by tumour necrosis factor α (TNFα) was studied.

2 IL-1α (1.5–6.0 ng ml^{-1}) and IL-1β (30–300 pg ml^{-1}), dose-dependently, stimulated PGE_2 formation, in 24 h cultures, with IL-β being the most potent agonist.

3 PHT (2.5–20 μg ml^{-1}) did not induce PGE_2 formation itself but potentiated IL-1α- and IL-1β-induced PGE_2 formation in the gingival fibroblasts in a manner dependent on the concentrations of both IL-1 and PHT.

4 IL-1β (0.1–1.0 ng ml^{-1}) induced release of [^{3}H]-arachidonic acid ([^{3}H]-AA) from prelabelled fibroblasts that was potentiated by PHT (20 μg ml^{-1}).

5 TNF-α ($\geqslant$ 0.01 μg ml^{-1}) significantly stimulated the biosynthesis of PGE_2 by a process that was potentiated by PHT.

6 Addition of exogenous arachidonic acid (AA) ($\geqslant$ 1 μM) caused an increase of PGE_2 formation in the fibroblasts that was not potentiated by PHT (20 μg ml^{-1}).

7 The results indicate that treatment with PHT results in upregulation of prostaglandin biosynthesis in gingival fibroblasts challenged with IL-1 or TNFα, at least partly due to enhanced level of phospholipase A_2 activity.

Keywords: Interleukin-1; tumour necrosis factor; phenytoin; gingival fibroblasts; prostaglandin E_2

Introduction

Phenytoin (PHT) is an anticonvulsant drug causing a number of side effects including skeletal, endocrine, immunological and connective tissue disturbances (Reynolds, 1975; Hassell, 1981; Yaari *et al.*, 1986). One of these, gingival overgrowth, is characterized by an increased amount of non-collagenous extracellular matrix associated with gingival inflammation (Nuki & Cooper, 1972; Dahllöf *et al.*, 1986). Gingival fibroblasts derived from PHT-induced gingival overgrowth are characterized by an increased synthesis of glycosaminoglycans (GAGs) compared to fibroblasts derived from normal gingiva (Dahllöf & Hjerpe, 1987). However, the mechanism(s) by which PHT treatment results in gingival overgrowth is still unclear.

Interleukin-1α (IL-1α) and IL-1β are closely related pleitropic cytokines produced in inflammatory lesions, mainly by macrophages (Dinarello, 1988). IL-1α and IL-1β have been found to stimulate bone resorption as well as prostaglandin E_2 (PGE_2) formation in bone cells (Gowen & Mundy, 1986; Lerner *et al.*, 1991) and in several other cells including gingival fibroblasts (Akahoshi *et al.*, 1988; Richards & Rutherford, 1990; Lerner & Modéer, 1991). IL-1β has also been reported to stimulate the biosynthesis of hyaluronic acid and proteoglycans in human gingival fibroblasts (Bartold, 1988a,b).

Recently, we demonstrated that gingival fibroblasts isolated after 9 months of PHT therapy produce much larger amounts of PGE_2 and PGI_2 *in vitro* when the cells were challenged with IL-1, as compared to the amounts produced by cells isolated before the start of the anticonvulsant therapy (Modéer *et al.*, 1992). The present investigation was undertaken to study whether addition of PHT to gingival fibroblasts *in vitro* results in a similar upregulation of IL-1-induced prostaglandin biosynthesis.

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Methods

Fibroblast cultures

Cultures of fibroblast cells were established from gingival biopsies obtained from three individuals (N-21, N-28, N-34), 9 to 11 years of age with no periodontal disease. The plan to take gingival biopsies was accepted by the Ethical Committee of Karolinska Institute. Minced pieces of the tissue were explanted to 25 cm^2 Falcon tissue culture flasks containing 5 ml of Eagle's basal medium (BME) medium. The fibroblasts were obtained by trypsinisation of the primary outgrowth of cells as previously described (Modéer *et al.*, 1982). The cells were grown in BME medium supplemented with sodium-glutamine (4 mmol l^{-1}), foetal calf serum (5%), penicillin (100 iu ml^{-1}) and streptomycin (100 μg ml^{-1}). The fibroblasts were incubated at 37°C in a humidified incubator gassed with 5% CO_2 in air and routinely passaged using 0.025% trypsin in phosphate-buffered saline (PBS) containing 0.02% EDTA. The cells used for the experiments proliferated in logarithmic phase between the 8th and 15th passage.

Prostaglandin production

Fibroblasts (2×10^4 cells) were seeded in Linbro multiwell dishes (24-well plate) in the presence of 5% foetal calf serum and grown for 48 h at 37°C. Then the cell layers were rinsed three times with BME medium without serum (37°C). Thereafter serum-free BME medium and the test substances recombinant human IL-1 (α;β) or tumour necrosis factor α (TNFα) were added in the presence or absence of phenytoin, 5.5-diphenylhydantoin (PHT). At different time points media were withdrawn, acidified to pH 3.5, frozen and stored at −20°C. In one series of experiments, the effect of addition of exogenous unlabelled arachidonic acid (AA) on PGE_2 bio-

synthesis was tested. In these experiments, the medium was withdrawn for analysis of PGE_2 after 24 h.

The amounts of PGE_2 in the media were determined by radioimmunoassays with a commercially available kit with [^{125}I]-PGE_2 as tracer (Gustafson *et al.*, 1986). After the experiments, the cells were detached with trypsin-EDTA in PBS and counted in a haemocytometer.

Analysis of [^{3}H]-arachidonic acid release

Fibroblasts (2×10^4 cells) were seeded in Linbro multiwell dishes (24-well plate) in medium containing 5% foetal calf serum and grown for 24 h at 37°C. Then the cell layers were rinsed once with serum-free medium and incubated in 0.5 ml serum-free medium per well containing 1 μCi ml^{-1} [^{3}H]-arachidonic acid ([^{3}H]-AA). After 24 h, the medium was withdrawn and the cells washed three times with serum-free medium (37°C). Subsequently, 0.5 ml HEPES-buffered (20 mmol l^{-1}) media, with different concentrations of IL-1β in the presence or absence of PHT, was added and the cells were further incubated for 24 h at 37°C. Samples of the media were withdrawn and analysed for ^{3}H in a scintillation counter. The ^{3}H-activity found in the supernatant represents free [^{3}H]-AA as well as ^{3}H-labelled metabolites.

Statistics

Student's *t* test (two-tailed) was used in the statistical analysis.

Chemicals

Eagle's basal medium, sodium-glutamine, foetal calf serum, penicillin and streptomycin were obtained from Flow laboratories, Irvine, Scotland; EDTA, 5.5-diphenylhydantoin and arachidonic acid from Sigma Chemical Co., St. Louis, U.S.A.; recombinant human interleukin-1α and β (specific activity 1.0×10^7 u mg^{-1}) from Boehringer, Mannheim, Germany; recombinant human tumour necrosis factor α (specific activity 2×10^7 u mg^{-1}) from Genzyme, Boston, U.S.A.; PGE_2 radioimmunoassay kit and [^{3}H]-arachidonic acid (specific activity 79.9 Ci mmol^{-1}) from Du Pont/New England Nuclear Chemicals, Germany.

Results

IL-1β, in 24 h cultures, dose-dependently stimulated PGE_2 formation in human gingival fibroblasts (Figure 1). Treatment of the cells with PHT (20 μg ml^{-1}) did not affect PGE_2 formation. When PHT (20 μg ml^{-1}) was added simultaneously with IL-1β, the stimulatory effect of IL-1β on PGE_2 formation was potentiated ($P < 0.07$) (Figure 1). The synergistic interaction between PHT and IL-1β (300 pg ml^{-1}) on PGE_2 formation in gingival fibroblasts was dependent on the concentration of the anticonvulsant drug (2.5– 20 μg ml^{-1}) (Figure 2). IL-1α ($\geq$ 1.5 ng ml^{-1}), in 24 h cultures, dose-dependently stimulated PGE_2 formation but was considerably less potent than IL-1β (Figure 3). When the fibroblasts were treated with IL-1α (1.5–6.0 ng ml^{-1}) together with PHT (20 μg ml^{-1}), the stimulatory effect of IL-1α on PGE_2 formation was potentiated ($P < 0.01$) by the anticonvulsant drug (Figure 3). All these experiments were performed with cells isolated from patient N-21. A similar upregulation of IL-1 (α, β)-induced PGE_2 formation by PHT was also observed in fibroblasts isolated from patients N-28 and N-34 (data not shown).

We have previously reported that the capacity to produce prostanoids is cell density-dependent (Lerner *et al.*, 1989). The effect of IL-1β on PGE_2 formation in fibroblasts was therefore tested at different cell densities ($7.5–30 \times 10^3$ cells cm^{-2}). At all cell densities tested, PHT potentiated IL-1β induced PGE_2 formation in the gingival fibroblasts (data not shown).

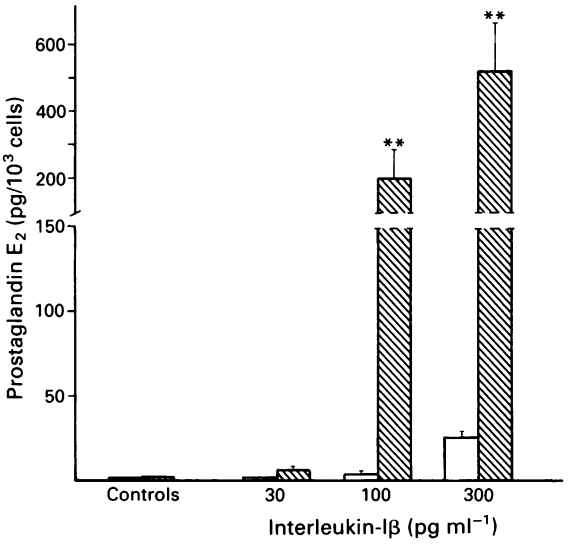

Figure 1 Effect of IL-1β (24 h) at different concentrations, in the absence (open columns) or presence of phenytoin (20 μg ml^{-1}) (hatched columns) on prostaglandin E_2 formation in human gingival fibroblasts (N-21). Cell density was 1.3×10^4 cells cm^{-2}. Mean value of triplicates with s.d. shown by vertical bars.

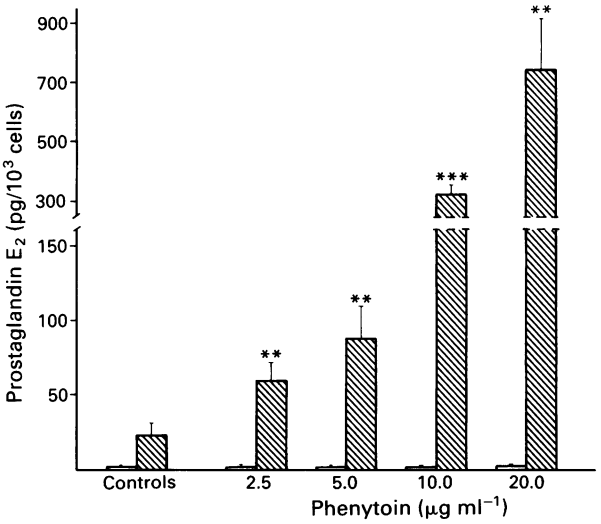

Figure 2 Effect of phenytoin (24 h) at different concentrations on prostaglandin E_2 formation in gingival fibroblasts (N-21), in the absence (open columns) or presence of (interleukin-1β) (IL-1β, 300 pg ml^{-1}) (hatched columns). Cell density was 1.2×10^4 cells cm^{-2}. Mean value of triplicates with s.d. shown by vertical bars.

In another series of experiments, the fibroblasts (N-21) were treated with TNFα (0.01–0.1 μg ml^{-1}) for 24 h, in the presence or absence of PHT (20 μg ml^{-1}). The TNFα-induced PGE_2 formation in fibroblasts was also potentiated ($P < 0.01$) by the anticonvulsant drug (Figure 4).

In a previous paper, we have shown that the capacity of IL-1β to stimulate PGE_2 formation in human gingival fibroblasts may, at least partially, be due to an enhanced release of arachidonic acid (Lerner & Modéer, 1991). We therefore studied whether PHT may potentiate IL-1β-induced release of [^{3}H]-AA from prelabelled fibroblasts. The cytokine IL-1β ($\geq$ 0.1 ng ml^{-1}), in agreement with previous findings (Modéer *et al.*, 1992) caused a dose-dependent, significant ($P < 0.05$) increase in the release of [^{3}H]-AA (Figure 5). When PHT (20 μg ml^{-1}) was added simultaneously with IL-1β ($\geq$ 0.3 ng ml^{-1}) the anticonvulsant drug potentiated ($P < 0.05$) the IL-1β-induced release of [^{3}H]-AA (Figure 5). When PHT was added in the absence of IL-1β, the drug itself did not increase the release of [^{3}H]-AA from the prelabelled fibroblasts.

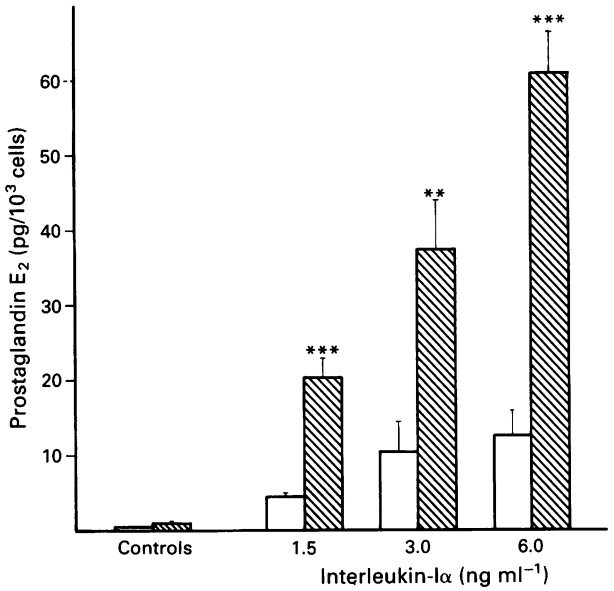

Figure 3 Effect of interleukin-1α (IL-1α; 24 h) at different concentrations, in the absence (open columns) or presence of phenytoin (20 μg ml^{-1}) (hatched columns), on prostaglandin E_2 formation in human gingival fibroblasts (N-21). Cell density was 1.3×10^4 cells cm^{-2}. Mean value of triplicates with s.d. shown by vertical bars.

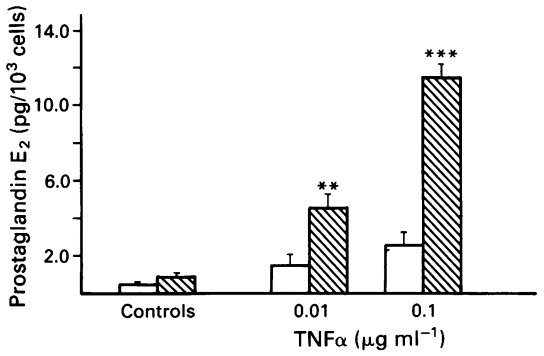

Figure 4 Effect of tumour necrosis factor α (TNFα; 24 h) at different concentrations, in the absence (open columns) or presence of phenytoin (20 μg ml^{-1}) (hatched columns), on prostglandin E_2 formation in human gingival fibroblasts (N-21). Cell density was 1.3×10^4 cells cm^{-2}. Mean value of triplicates with s.d. shown by vertical bars.

In one series of experiments we also examined the effect of addition of different concentrations of exogenous non-labelled AA upon the biosynthesis of PGE_2 in gingival fibroblasts in the presence or absence of PHT. In the absence of PHT, exogenous AA (> 1.0 μM) caused a dose-dependent increase in PGE_2 formation (Figure 6). Addition of AA in the presence of PHT (20 μg ml^{-1}) did not result in an increased PGE_2 formation in 24 h cultures of fibroblasts compared to the cultures not treated with PHT (Figure 6).

Discussion

In agreement with previous observations (Lerner & Modéer, 1991; Modéer *et al.*, 1992) we have found that IL-1α and IL-1β, in 24 h cultures, dose-dependently stimulate PGE_2 formation in human gingival fibroblasts, with IL-1β being the more potent agonist. The novel finding in the present study is that the anticonvulsant drug PHT, which by itself does not affect prostanoid biosynthesis, potentiates IL-1α- and IL-1β-induced PGE_2 biosynthesis in human gingival fibroblasts *in vitro*. The PHT-induced upregulation of PGE_2 formation was seen in cells challenged not only with IL-1α and IL-1β but also with TNFα indicating that the level of upregulation of prostaglandin biosynthesis is not related to receptor number or receptor affinity of IL-1 but rather a step distal to receptor ligand interaction.

Recently we have shown that gingival fibroblasts isolated after 9 months of PHT therapy produce much larger amounts of PGE_2 and PGI_2 *in vitro* as compared to the amounts produced by fibroblasts isolated before the treatment with the anticonvulsant drug (Modéer *et al.*, 1992). The data in the present paper demonstrate that the effect on fibroblast prostaglandin production caused by treatment with the anticonvulsant drug also can be seen by direct addition of PHT to fibroblasts isolated from untreated subjects.

The upregulation of PGE_2 formation in gingival fibroblast in the presence of PHT is probably, at least to some extent,

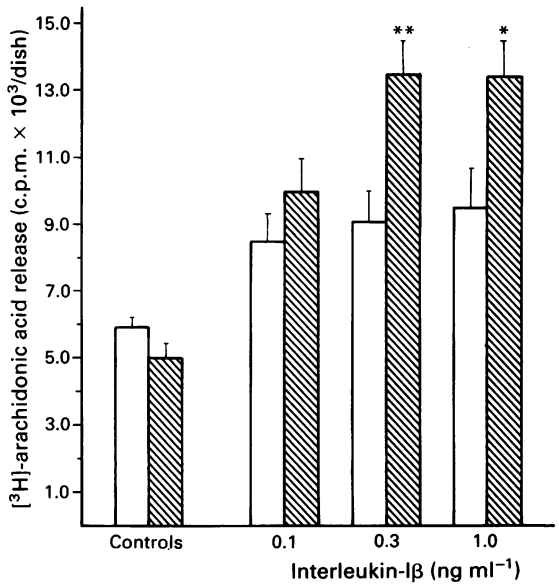

Figure 5 Effect of interleukin-1β (IL-1β; 24 h) at different concentrations, in the absence (open columns) or presence of phenytoin (20 μg ml^{-1}) (hatched columns), on the release of [^{3}H]-arachidonic acid ([^{3}H]-AA) and ^{3}H-labelled metabolites from gingival fibroblasts (N-21) prelabelled with [^{3}H]-AA (1 μCi ml^{-1}). Cell density was 1.2×10^4 cells cm^{-2}. Mean value of triplicates with s.d. shown by vertical bars.

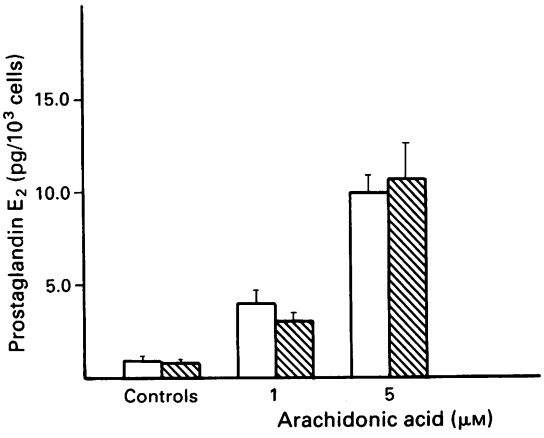

Figure 6 Effect of different concentrations of exogenous unlabelled arachidonic acid added to the cells (24 h) in the absence (open columns) or presence of phenytoin (20 μg ml^{-1}) (hatched column), on the prostglandin E_2 formation in human gingival fibroblasts (N-28). Cell density was 1.0×10^4 cells cm^{-2}. Mean value of triplicates with s.d. shown by vertical bars.

due to an increased phospholipase A_2 (PLA_2) activity, resulting in increased release of arachidonic acid. This assumption is based on the findings that IL-1-induced significantly higher release of [^{3}H]-AA in the presence of PHT. The enhancement of PLA_2 activity also seems to occur as a consequence of PHT medication since we earlier demonstrated that the release of [^{3}H]-AA was significantly higher in fibroblasts isolated during PHT medication in comparison to the level in gingival fibroblasts isolated before the start of the PHT therapy (Modéer *et al.*, 1992). The enzyme PLA_2 is calcium ion-dependent and may be influenced by the anticonvulsant drug, since we have reported that PHT affects the intracellular calcium ion level in gingival fibroblasts *in vitro* (Modéer *et al.*, 1991).

Addition of exogenous AA to the gingival fibroblasts results in enhanced PGE_2 formation. When the cells were treated with exogenous AA and PHT simultaneously, the PHT-treated cells did not produce larger amounts of PGE_2 than the fibroblasts in the absence of PHT. This finding indicates that the upregulation of the IL-1-induced PGE_2 formation seen in the presence of PHT is not due to upregulation of cyclo-oxygenase activity but to enhancement of PLA_2 activity.

However, we found earlier that an enhanced level of cyclo-oxygenase activity also contributes to the upregulation of PGE_2 formation, found in fibroblasts isolated during PHT medication (Modéer *et al.*, 1992). This indicates that upregulation of cyclo-oxygenase activity by PHT may require long term exposure to the drug or that a metabolite of PHT rather than the drug itself may be involved in the stimulation of cyclo-oxygenase.

The PHT-induced potentiation of IL-1 induced release of [^{3}H]-AA in the fibroblasts is less than would be expected compared to the large degree of PHT-induced enhancement of IL-1-stimulated PGE_2 biosynthesis. This discrepancy may indicate that PHT also affects the metabolism of other metabolites of arachidonic acid (thus favouring PGE_2 biosynthesis) or that the drug may inhibit the degradation of PGE_2. Another possibility could be that PHT affects the reacylation of arachidonic acid. The fact that PHT did not potentiate TNFα-induced PGE_2 formation as much as IL-1-induced PGE_2 biosynthesis argues against the view that PHT affects arachidonic acid metabolism at several levels. Rather, the preferential large degree of upregulation of IL-1-induced PGE_2 biosynthesis indicates that PHT specifically potentiates the mechanism of action for IL-1-induced PGE_2 formation.

Our observations in the present study are in contrast to a report by Katsumata and co-workers who showed that PHT inhibits the production of 6-keto-$PGF_{1\alpha}$, the stable metabolite of prostacyclin, in mice thymocytes (Katsumata *et al.*, 1982). We do not have any simple explanation for the conflicting results although the discrepancy may be due to species or cell type differences.

Whether the upregulation of prostaglandin synthesis induced by PHT plays an important role in the pathogenesis of the drug-induced gingival overgrowth is so far unclear. The consequence of the upregulation of prostaglandin formation in gingival fibroblasts due to PHT on extracellular matrix synthesis should be further studied, since it has been reported that hyaluronic acid synthesis as well as GAGs synthesis in fibroblasts may be regulated by PGE_2 (Bartold, 1988a; Karlinsky & Goldstein, 1989). This is specially relevant in light of earlier reports showing that PHT-induced gingival overgrowth represents a tissue with an increased amount of hyaluronic acid as well as of GAGs as compared to normal gingival tissue (Dahllöf *et al.*, 1986).

It has been reported that the cyclo-oxygenase inhibitor, acetylsalicylic acid, reduces the incidence of foetal cleft palate in CD-1 mice treated with PHT (Wells *et al.*, 1989). In animals treated with both PHT and 12-O-tetradecanoylphorbol-13-acetate (TPA) the anticonvulsant drug-induced embryopathy was enhanced, indicating that protein kinase C may be involved in the potentiation (Wells & Vo, 1989).

In conclusion, this study demonstrates that PHT potentiates the prostaglandin biosynthesis in gingival fibroblasts, challenged with IL-1 or TNFα, partly due to enhanced PLA_2 activity.

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